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Fluorescent Protein Tracers: The Unreacted Fluorescent Material in Fluorescein Conjugates and Studies of Conjugates with other Green Fluorochromes

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Summary. Protein conjugates with fluorescein isocyanate and isothiocyanate contain unreacted fluorescent material (U.F.M.) which contributes to specific immunological staining but is troublesome because it also stains non-specifically. Attempts have been made to find an alternative to fluorescein isocyanate and isothiocyanate conjugates, with similar green fluorescence but free from U.F.M. and more easily prepared. Conjugates were made with fluorescein and certain derivatives of fluorescein in the form of the acid chlorides, 'reactive' dyes, a dichlorocyclopropyl derivative of a stilbene sulphonic acid (R 8065/1, Geigy), and 3-hydroxypyrene 5-8-10 trisulphonic acid; none proved satisfactory. At the present time, green fluorescent protein tracers are probably best prepared from fluorescein isothiocyanate.

The properties and nature of the U.F.M. in fluorescein conjugates have been investigated; it accounts for nearly half of their fluorescence after full dialysis and two extractions with tissue powder. Extraction with powdered activated charcoal removes the U.F.M. almost completely but the staining intensity of the conjugates is so reduced that the procedure cannot be recommended for routine use. This property of charcoal provides a simple means of testing fluorescein conjugations: a sample from a satisfactory conjugate should retain substantial fluorescence after such extraction.

INTRODUCTION

Despite the disadvantages of fluorescein as a protein label (discussed previously, Chadwick, McEntegart and Nairn, 1958) fluorescein conjugates, prepared either from fluorescein isocyanate (Coons and Kaplan, 1950) or from the isothiocyanate (Riggs, Seiwald, Burckhalter, Downs and Metcalf, 1958), have been extremely valuable tools in immunological tracing. They can be replaced for many purposes by conjugates with Lissamine Rhodamine B 200 (RB 200) which have practical advantages (Nairn, Fraser and Chadwick, 1959; Fraser, Nairn, McEntegart and Chadwick, 1959) and doubtless the value of further alternatives suggested by other workers (e.g. Uehleke, 1958) will be established. For some purposes, however, fluorescein conjugates are likely to remain useful. They are perfectly satisfactory for staining bacterial smears and suspensions, in fact in every situation where colour contrast with the background, e.g. tissue autofluorescence, is unimportant. In some types of fluorescent tracing they may provide maximum contrast with background autofluorescence, as with the red fluorescence of chlorophyll in the green parts of plants. The colour contrast with RB 200 conjugates is also ideal for double tracing experiments.

It is unfortunate that fluorescein conjugates have two major disadvantages which have not yet been overcome: their preparation is complicated and difficult, and when used as immunological tracers, they are prone to cause non-specific staining of organisms and tissues. The present study reports unsuccessful attempts to simplify the preparation of conjugates with green fluorescence, using fluorescein and other green fluorochromes. Non-specific staining by fluorescein conjugates is partly attributable to the presence of unreacted fluorescent material (U.F.M.) (Chadwick, Nairn and McEntegart, 1959): an investigation of the nature and properties of this material is described.

METHODS

INVESTIGATION OF DIFFERENT CONJUGATION METHODS

Protein conjugation with fluorescein isocyanate and isothiocyanate has been successful in practice because it is accomplished without appreciable reduction of the fluorescence intensity of the dye and the linkage is sufficiently stable for tracing purposes. The isothiocyanate has the advantages that it is more stable than the isocyanate and is a little easier to prepare, liquid thiophosgene being used instead of the gaseous phosgene. Fluorescein isothiocyanate has recently become available commercially in U.S.A. but is very expensive.

We have attempted to obtain a satisfactory green fluorescent conjugate more simply in two ways

(*a*) Fluorescein or its derivatives containing either carboxylic or sulphonic acid groups were treated with PCl_5 to produce acid chlorides. These were combined directly with serum proteins. The procedure was the same as for RB 200 conjugations (Chadwick *et al.*, 1958). The following substances were used:

1. Fluorescein, which contains a carboxylic acid group and can be treated directly with PCl_5.
2. Calcein, bought commercially (L. Light & Co.) or prepared by treating fluorescein with iminodiacetic acid and formaldehyde (Diehl and Ellingboe, 1956).
3. Glycine or taurine derivatives of fluorescein, prepared by substituting glycine or taurine for iminodiacetic acid in the above method.

(*b*) The other green fluorochromes used were:

1. The 'reactive dyes' produced by treating proflavine (2 : 8-diaminoacridine) or rheonine [2-amino-8-dimethylamino-5 (p-dimethylaminophenyl) acridine] with cyanuric chloride. The combination of such reactive dyes with proteins has been described by Hess and Pearse (1959).
2. A dichlorocyclopropyl derivative of a stilbene sulphonic acid (R 8065/1, Geigy & Co. Ltd.) which may be conjugated directly with protein.
3. 3 hydroxypyrene-5-8-10 trisulphonic acid was converted to the sulphonyl chloride and conjugated to serum proteins (Uehleke, 1958).

U.F.M. IN FLUORESCEIN CONJUGATES

U.F.M. in fluorescein conjugates is only partly removed by dialysis; a proportion remains strongly adsorbed on the serum proteins. Treatment of dialysed conjugates with acetone-dried tissue powders (Coons, 1956) extracts some of this adsorbed dye. The following experiment was designed to determine the proportion which remains. Two samples

of crystallized bovine albumin (Armour) were conjugated in the usual way, one with fluorescein isocyanate* and the other with isothiocyanate.† After dialysis and two extractions with acetone-dried tissue powder, they were each shaken for 1 hour with powdered activated charcoal (British Drug Houses, 1 mg./mg. protein); extraction with charcoal removed the adsorbed dye almost completely (Chadwick *et al.*, 1959). Samples of the conjugates were taken at each stage for total nitrogen, fluorimetric and spectrophotometric absorption measurements. Nitrogen measurements were required to correct the fluorimetric and absorptiometric values to the same protein concentration. Absorptiometry was performed (*a*) as a direct measure of concentration of fluorescent material and (*b*) to detect changes in wavelength of maximum light absorption (λ max) which would suggest differential extraction of certain components.

In order to determine the nature of the U.F.M., the following preparations were studied:

Sample 1. The crystallized product from a mixture of fluorescein isocyanate and buffered saline (pH 9) which had been stirred overnight, precipitated by acidification and redissolved in alcohol.

Sample 2. The material obtained by evaporation to dryness of dialysates from fluorescein isocyanate and isothiocyanate conjugates.

Sample 3. The alcohol extract of a tissue powder which had been shaken for an hour with a freshly dialysed fluorescein isocyanate conjugate and recovered by centrifugation for 30 minutes at 10,000 rev./min. An unsuccessful attempt was made to extract used activated charcoal with alcohol in a similar manner.

Paper chromatography of these preparations was carried out with phenol saturated with water, and with 25 per cent ethanol/5 per cent NH_4OH (1 : 1) as solvents. The results were compared with paper chromatograms of the sample of aminofluorescein from which the fluorescein isocyanate used in these experiments had been prepared.

ROLE OF U.F.M. IN HISTOLOGICAL STAINING BY CONJUGATES

Anti-rabbit globulin serum (A.R.G.), prepared in a goat, was used for these experiments. A mixture of the serum with *Sample 1* fluorescent material was made by adding 2.5 mg. of the dye and 2 ml. of buffered saline (pH 9) to each ml. of serum and stirring overnight. The product was dialysed until no further fluorescence appeared in the dialysate and it was then extracted twice with acetone-dried tissue powder. Its histological staining properties were compared with those obtained with a mixture of A.R.G. serum and aminofluorescein prepared in the same way. Comparison was also made with the staining potency of conjugated A.R.G. serum both before and after extraction with charcoal. Staining tests were made on a variety of preparations, but mainly on pig tissue fresh frozen sections previously treated with organ or tissue specific antisera prepared in rabbits (e.g. anti-pig kidney and anti-pig reticulin); a few tests were made on acetone-fixed *klebsiella* smears coated with specific rabbit antiserum.

RESULTS

ACID CHLORIDE CONJUGATIONS

Successful chemical attachment to protein was achieved in each case as judged by the

* Prepared throughout the present experiments from isomer I aminofluorescein.
† Prepared from aminofluorescein of mixed isomers I and II.

persistence of fluorescence after extraction with charcoal. The conjugates with fluorescein, and with the glycine and taurine derivatives, showed only poor fluorescence. The calcein conjugate, on the other hand, was brightly fluorescent although it compared unfavourably with a parallel fluorescein isocyanate conjugate both as a plasma tracer and as an immunological stain. Calcein-conjugated A.R.G. serum had only about 3/4 the staining power of a similar fluorescein isocyanate conjugate; there was no difference in their antibody titres. The possibility that the greater fluorescence intensity of the isocyanate conjugate may have been due to the adsorbed dye, was excluded by charcoal extraction of the sera: this reduced the fluorescence in both types of conjugate by a proportionately similar amount and the isocyanate conjugate remained brighter. It seems likely that the diminished fluorescence in the acid chlorides was due to reaction between the PCl_5 and other parts of the fluorescein molecule to produce some derivatives with reduced fluorescence.

TABLE I

EFFECT OF TISSUE POWDER AND CHARCOAL EXTRACTION ON DIALYSED FLUORESCEIN-CONJUGATED BOVINE ALBUMIN

Fluorescein conjugate (1 mg. protein/ml.)	*Physical characteristics*	*Extractions*			
		Nil	*1st tissue powder*	*2nd tissue powder*	*Activated charcoal*
Isocyanate	Wavelength of maximum light absorption (λ max)	495 mμ	490 mμ	490 mμ	485 mμ
	Optical density at the λ max value above	1.741	1.080	0.938	0.408
	Fluorimetric value*	100	60.5	50.0	22.6
Isothiocyanate	λ max	495 mμ	490 mμ	490 mμ	485 mμ
	Optical density at the λ max value above	2.62	2.21	1.76	1.27
	Fluorimetric value*	100	74.0	58.0	41.7

* Primary illumination by mercury arc lamp filtered to transmit ultraviolet of wavelength 290–400 mμ.

OTHER FLUOROCHROMES

Protein conjugates with the 'reactive dyes' lost most of their fluorescence when extracted with charcoal; their histological staining power was almost negligible.

Conjugates prepared from the Geigy dye R 8065/1 stained specifically and brilliantly after charcoal extraction, but unfortunately the fluorescence was a blue-green colour scarcely distinguishable from tissue autofluorescence; moreover, they were highly toxic when injected in tracer doses into rats.

Serum conjugates with the dye 3-hydroxypyrene-5-8-10 trisulphonic acid had about the same fluorescence intensity as fluorescein isocyanate conjugates, but were unsatisfactory because their blue-green fluorescence was similar to that of tissue autofluorescence.

U.F.M. IN FLUORESCEIN CONJUGATES

Table 1 shows the effect of extraction by tissue powder and by charcoal on λ max, optical density at λ max, and fluorescence emission of crystallized bovine albumin conjugated with fluorescein isocyanate or isothiocyanate. The measurements indicate progressive loss, with each successive extraction, of unreacted fluorescent material from the dialysed conjugates. Extraction with tissue powder removes about 70 per cent of the unreacted fluorescent material present in the conjugates after dialysis: the remaining 30 per cent was removed by the activated charcoal. The small change of λ max as the extractions were performed can be readily explained on the assumption that the dye in chemical combination with the protein has a different λ max from the extractable fluorescent material. In the fluorescein isocyanate conjugate comparison of the values after the 2nd tissue powder extraction and after the charcoal extraction shows that only about 50 per cent of the dye in the tissue-extracted material is in chemical combination with the protein. In the isothiocyanate conjugate about 65 per cent of the dye, still present after two extractions with tissue powder, is in chemical combination.

TABLE 2

STAINING BY FLUORESCEIN CONJUGATES, FLUORESCENT MIXTURES AND AQUEOUS FLUORESCENT SOLUTIONS

	Dialysed fluorescein-conjugated A.R.G. serum		*Dialysed A.R.G. serum mixtures*		*Aqueous solutions*	
			Sample 1 fluorescent material	*Amino-fluorescein*	*Sample 1 fluorescent material*	*Amino-fluorescein*
Extraction	Tissue powder and charcoal	Tissue powder	Tissue powder	Tissue powder	Nil	Nil
Specific staining	++	++++	++	Minimal	Nil	Nil
Non-specific staining	(+)	++	+	Minimal	Very intense, diffuse	Intense

In order to compare the above results with those obtained by ethyl acetate extraction (Dineen and Ada, 1957) two such extractions were carried out on a sample of dialysed conjugated serum. The ethyl acetate removed about 75 per cent of the unconjugated fluorescent material remaining after dialysis, i.e. much the same proportion as that removed by two extractions with tissue powder.

The paper chromatography studies on *Samples 1, 2 and 3* (page 365) of the fluorescent material revealed two components. The major component 'A' in *Samples 1 and 2* had, in two different solvents, an R_f value similar to that of aminofluorescein. The proportion of this component was much less in *Sample 3*. The minor component 'B' had a lower R_f value than 'A' in both solvents. Component 'A' is probably aminofluorescein which might have been produced by simple hydrolysis of fluorescein isocyanate. Component 'B' might be the 'ureido' derivative from combination of aminofluorescein with its isocyanate, or other similar derivative. Some of the possible derivatives have a common empirical formula and we have been unable to obtain definite evidence in favour of any particular compound.

STAINING PROPERTIES OF U.F.M.

Comparisons were made of staining by the following materials (Table 2): fluorescein-conjugated A.R.G. serum with and without charcoal extraction; mixtures of antiserum with *Sample 1* fluorescent material and with aminofluorescein; aqueous solutions of *Sample 1* and of aminofluorescein. The fluorescein conjugate, before extraction with charcoal, was more powerful than the fluorescent mixture in both specific and non-specific staining. This result is understandable because the conjugated material contains both chemically combined and physically adsorbed dye in roughly equal proportions; the fluorescent mixture contains adsorbed dye only. Extraction of the conjugate with charcoal removed the adsorbed dye and reduced staining, especially non-specific staining. Although aqueous solutions of both dyes gave intense non-specific fluorescent staining, the aminofluorescein-serum mixture after dialysis and tissue powder extraction had negligible fluorescence or staining potency. This is presumably attributable to weak adsorption of aminofluorescein by serum proteins so that it is largely removed by dialysis and tissue powder extraction.

The results indicate that the principal staining component in serum mixtures with *Sample 1* fluorescent material is component 'B' and not aminofluorescein. It also seems likely that component 'B' is the adsorbed dye in fluorescein-protein conjugates which remains after dialysis and tissue powder extraction, i.e. the fluorescent material removed by the final charcoal extraction. This has not, however, been proved as we were unable to recover the dye from the charcoal. It seems reasonable to assume that during staining by conjugates that have not been extracted with charcoal, the adsorbed dye is transferred non-specifically from the serum proteins to the tissue proteins and thus contributes to the staining.

Serial staining experiments were carried out daily for 10 days after preparation of the conjugates and mixtures, which were stored at 0° C. between experiments. Non-specific staining remained minimal with the charcoal-extracted conjugate; the conjugate and the *Sample 1* fluorescent mixture, which had been extracted with tissue powder, showed a small increase in non-specific staining during the first 2 or 3 days. This increase in staining was possibly due in part to 'desorbed' dye in solution; its failure to progress could be the result of the establishment of an equilibrium between the dye adsorbed and in aqueous solution, after tissue powder extraction.

DISCUSSION

CONJUGATION WITH FLUORESCEIN DERIVATIVES AND OTHER FLUOROCHROMES

Neither the acid chlorides of fluorescein and its derivatives nor the other fluorochromes tried were satisfactory alternatives to fluorescein isocyanate or isothiocyanate. The acid chloride conjugates were less fluorescent than the isocyanate or isothiocyanate conjugates. The only new fluorochromes giving stable conjugates of high fluorescence intensity were R 8065/1 (Geigy) and 3-hydroxypyrene-5-8-10 trisulphonic acid. Uehleke has suggested that the latter may be valuable as a protein label. Our conjugates with either fluorochrome had an unsatisfactory blue-green fluorescence which would limit their usefulness.

U.F.M. IN FLUORESCEIN ISOCYANATE AND ISOTHIOCYANATE CONJUGATES

Substantial amounts of U.F.M. are present in fluorescein isocyanate and isothiocyanate conjugates, even after exhaustive dialysis and two tissue powder extractions. Extraction with ethyl acetate has also failed to remove this strongly adsorbed U.F.M. which can,

however, be removed almost completely by charcoal extraction. The quantitative experiments on isocyanate conjugates with crystallized bovine albumin suggest that, after the two tissue powder extractions, chemically combined dye and adsorbed dye are present in about equal amounts. This might explain the superiority of isothiocyanate over isocyanate already demonstrated in comparative staining experiments by Marshall, Eveland and Smith (1958.) If this observation is confirmed, isothiocyanate is likely to be superior to isocyanate in the preparation of fluorescein conjugates. The U.F.M. has been shown to consist of at least two components: 'A', probably amino-fluorescein, seems to be less strongly adsorbed and therefore present in only small amounts after complete dialysis and tissue powder extraction; 'B', an unidentified fluorescent compound, is an important staining component in conjugates which have not been extracted with charcoal.

The U.F.M. contributes materially to both specific and non-specific staining by fluorescein isocyanate and isothiocyanate conjugates; its removal by charcoal diminishes staining intensity but increases specificity. The reduction in staining is too great for charcoal extractions to be recommended for routine use in the preparation of fluorescein conjugates, though the improvement of staining specificity is sometimes of practical value. Non-specific staining increases with ageing of conjugates. This suggests that U.F.M. may be slowly desorbed from the protein into the aqueous phase; some type of equilibrium distribution of dye between protein and aqueous phase may be established after a few days. The histological problems presented by non-specific staining will be relatively greater when specific staining is weak, e.g. when only low titre antiserum is available. The ratio of U.F.M. to conjugated specific antibody in antiserum conjugates will be reduced by separating the antibodies from the other serum proteins. It follows that some such procedure as ammonium sulphate fractionation of conjugated antisera to obtain globulin, or better, γ-globulin, is desirable for the preparation of conjugates of maximum staining specificity.

These results do not imply that non-specific staining is due only to U.F.M.; protein-protein interactions and unwanted tissue antibodies in the conjugate are also important factors (Coons, 1956). Non-specific staining with charcoal-extracted conjugates was presumably due to these factors. It is noteworthy that the ability of charcoal to remove the strongly adsorbed U.F.M. from proteins provides a very simple practical test of successful conjugation: substantial fluorescence should be retained in a charcoal-extracted sample if adequate chemical conjugation has been achieved.

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